Research article

Genetic defense analysis of tomatoes in response to early blight disease, *Alternaria alternata*

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ABSTRACT

Early blight disease of tomato is one of the most devastating biotic stresses worldwide, and in Iran, *Alternaria alternata* is one of the most predominant species causing the disease. In the current study, a diverse collection of 35 tomato genotypes and implication of 5 SlWRKYs and 7 PR genes as well as enzymatic activity were evaluated on resistant and susceptible cultivars through real-time polymerase chain reaction at transplanting and maturing stages and by measuring product formation using spectrophotometry. The results indicated that the expression of these antifungal genes in 14 genotypes at two growth stages after inoculation with *A. alternata* highly enhanced by 1–50-fold. There was also significant upregulation of WRKYs and PRs genes among the resistant tomato varieties in comparison to susceptible and control varieties at both stages. These findings demonstrate the varieties that showed increased or decreased SlWRKY1 expression also displayed similar changes in the expression of PR1 and PR2 genes. Furthermore, the differential expression patterns of SlWRKY1 and SlWRKY11 were consistent with PR7 and PDF1.2 expression patterns. The analysis of enzymatic activity of PR2 and PR3 proteins, β-1,3-glucanase, and chitinase showed the highest level of activity in resistant inoculated genotypes against *A. alternata*. Therefore, the current findings suggest the possible involvement of these transcription factors in the increased expression of PR genes in response to *A. alternata* infection.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop around the world, since it has been widely adopted by many countries as a valuable part of their daily alimentation and even culinary traditions. In Iran, tomato is recognized as one of the most laborious crops, with an average labor cost of 1093.2 h/ha (Jadidi et al., 2012; Chaudhary and Atamian, 2017).

Among the various diseases impacting tomato crops worldwide, the early blight (EB) is considered the most devastating one. It is caused by several species of fungi belonging to the genus *Alternaria*, including *A. solani* and *A. alternata* (Adhikari et al., 2017; Nasr Esfahan, 2018a). Particularly, *A. alternata* is capable of infecting tomato crops at all stages of plant development, causing considerable losses during crop harvesting (Bashir et al., 2016). The sheer scope of the catastrophic impact of *Alternaria* species in tomato cultivation extends to Iran, with *A. alternata* being considered as the dominant species among them

given that it causes significant losses and deteriorates the nutritive value of tomato (Piquerez et al., 2014; Nasr Esfahani, 2018a, b). Furthermore, although the application of several fungicides has been recommended to fight this disease, indiscriminate use of fungicides considerably increases hazards to humans and the environment (Roylawar et al., 2015; Nasr Esfahani, 2018b). Therefore, the easiest as well as the most economical, harmless, and effective approach is to develop and/or select sufficiently resistant tomato crops that are capable of producing satisfactory yield, even within heavily pathogen-infested environments (Ahmad et al., 2013; Nasr Esfahan et al., 2017b).

Recently, only a limited number of studies paid attention to the analysis of EB disease with the aim of identifying the molecular basis of the plants' natural defense mechanisms (Adhikari et al., 2017). When attacked by pathogens, plants defend themselves through structural and biochemical responses, with the first line of defense preventing the colonization of pathogens in the surrounding tissues, including the formation of structural barriers and secretion of antimicrobial

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compounds, cutin waxes, phytoanticipins, and lignin (Ali et al., 2017; Nasr Esfahani et al., 2017a). Additionally, there are numerous plant responses in active or induced defense mechanisms, such as lignification, hypersensitivity response, and the production of phytoalexin and pathogenesis-related proteins (Abdelrahman et al., 2019; Yang et al., 2015).

Notably, transcriptome studies have determined pathogenesis-related proteins to be effective against multiple stress conditions, making the creation of various stress-tolerant crops possible (Upadhyay et al., 2014; Ali et al., 2017). For example, upon invasion and subsequent colonization by fungal infection, plants produce chitinase, a pathogenicity-related (PR3) protein, in response to chitin, the major component of most fungal cell walls (Adhikari et al., 2017). Although defense mechanisms vary in terms of methodology and complexity across different cultivars, the antifungal effect of chitinases and other hydrolytic enzymes has been determined against *A. solani* and other biotic stress (Adhikari et al., 2017). Further research also concluded that the genes responsible for the production of pathogenesis-related proteins have significantly improved resistance against various pathogens in different crops (McNeece et al., 2019; Upadhyay et al., 2014).

In order to perform a detailed analysis of plant defense responses, it is important to identify key regulatory factors. Through the analysis of proteins binding to sequence-specific DNAs within various plant species, a number of transcription factors (TFs) coordinating crosstalk signals have been identified and shown to be involved in the activation or inhibition of target genes, either alone or via interactions with other proteins (Shinde et al., 2018). Because of their tightly regulated transcriptome, plants possess the ability to rapidly reshape it in response to various types of stress (Shinde et al., 2018).

Tomato has 83 SlWRKY genes, which form part of the defense responses to biotic and abiotic stresses (Bai et al., 2018). Many of these WRKYs genes function in tomato as positive regulators of plant responses to biotic stresses, whereas a minority of these function as negative regulators. WRKY proteins interact with W-boxes located in the promoter regions of several plant defense genes. These boxes are found in clusters within the short stretches of the promoters, which suggest that potential synergetic interactions exist between different WRKYs (Shinde et al., 2018; Aljaafria et al., 2017).

Several studies revealed that the expression of a number of WRKY genes was significantly altered by the invasion of pathogens. For example, overexpression of the *Solanum pimpinellifolium* WRKY1 resulted in resistance to *Phytophthora nicotianae* in tobacco and *Phytophthora infestans* in tomato. Additionally, WRKY31 and WRKY33 were shown to increase the tolerance of Arabidopsis *wrky33* mutant to *Botrytis cinerea* pathogen. Moreover, overexpression of the grape (*Vitis quinquangularis*) WRKY52 gene, a homolog of SlWRKY53/54, in Arabidopsis enhanced resistance to *Golovinomyces cichoracearum*, but increased susceptibility to *B. cinerea*, which leads to increased expression of SA pathway-related genes and subsequent apoptosis (Shinde et al., 2018).

Therefore, the objective of this study was to determine the natural defense responses of a number of exotic and domestic, resistant and susceptible tomato cultivars to *A. alternata* for the very first time, and to explore the molecular basis underlying these defense mechanisms in resistant ones, with the aim of presenting these resistant genotypes as viable options for tomato cultivation within the agricultural industry.

2. Materials and methods

2.1. Plant material and growth conditions

A seed collection of 35 commercial exotic and domestic tomato hybrids, as well as inbreeding lines belonging to several seed companies were chosen for this assay (Table 1). No screening for resistance to *A. alternata* was previously performed for these tomato genotypes. Screening experiments were conducted in the greenhouse and laboratory of the Agricultural and Natural Resource Research and Education

center of Esfahan, AREEO, Iran, in 2017–2018.

2.2. Fungal culture inoculum preparation and plant pathogenicity

Active isolates of *A. alternata* were obtained from the above-mentioned center from the leaves of infected tomato plants. The *A. alternata* isolates were cultured in PDA medium and stored for 15 days at 25 °C, before the cultures were induced to sporulation as described by Shahin and Shepard (1979). Tomato genotypes were screened for EB resistance in a completely randomized design, with three replications (n = 10 plants each). Inoculum and inoculation preparations of the fungi were performed as described by Nasr Esfahani et al. (2018a). Inoculated and non-inoculated plants were kept at 27–28 °C and subjected to a 16 h photoperiod and 65% relative humidity. Two screening assays were carried out at transplanting stages (6 weeks) and maturing stages (12 weeks) separately. The conidial suspension (10^5 spores/ml) was sprayed on the tomato plants' leaves and repeated three times, in a 3-day interval and under glasshouse conditions.

Then, 20 days after the inoculation in independent experiments and after the appearance of the disease symptoms, the plants were evaluated in both assays based on infection percentage and the disease scoring scales. Infections were assessed based on infection percentage (0–100) and the severity was expressed using six scoring scales, namely: 0, 5, 10, 25, 75, and 100 percent (Hayat Moghaddam et al., 2011, Nasr Esfahani et al., 2018a). The data were first converted with Asin $\sqrt{x}$ + 0.5 for homogeneity and then, variance analysis was performed using SAS software, version 9.4. The least significant difference (LSD) is used to compare the means. Statistical significance of differences was accepted at $P < 0.01$ (SAS Institute, 2013).

2.3. Leaves sample collection

To analyze some defense-related enzymes and genes, 14 contrasting susceptible and resistant genotypes of tomato (Table 1) were selected based on the results of screening test (Fig. 1, A-B). Following re-inoculation at the transplanting and maturing stages, the third leaf of selected genotypes from control (non-inoculated) and inoculated treatments were separately collected in aluminum foils and stored in −80 °C for RNA isolation. Each of these samples was represented by a bulk of five individual plants ground together in liquid nitrogen. The experiment was repeated three times (Upadhyay et al., 2014).

2.4. RNA extraction and cDNA synthesis

Total RNA extraction was performed using Iraizol kits (RNA Biotech, Co, Isfahan, Iran) according to the manufacturer's manual. Thus, 100 mg of leaf tissue was powdered while immersed in liquid nitrogen, and 1 ml of RNA extraction buffer was added to it. The mixture was then incubated at room temperature (23 °C) for 5 min followed by addition of 200 μl of chloroform prior to being vigorously shaken for 10–15 s. The mixture was then incubated at room temperature for 5 min followed by centrifugation at 12 000 g for 5 min in a 4 °C refrigerated centrifuge, which resulted in the formation of two separate layers. The top clear layer, which contained RNA, was carefully separated and transferred to a nuclease-free tube followed by the addition of 1 ml of ice-cold 100% ethanol and incubation at −20 °C for 15–20 min. Finally, the tube was centrifuged for 8 min at 12 000 g in a refrigerated centrifuge. The supernatant was then discarded and 50 μl RNase-free water was added to the dried precipitate. RNA concentration and purity were determined with Nano spectrophotometer (Epoc, Biotech, USA) and electrophoresis using a 1.2% agarose gel. Extracted RNA was treated with DNase I (RNA Biotech, Co) and cDNA synthesis was performed using the RB MMLV Reverse Transcriptase Kit (RNA Biotech, Co) according to the manufacturer's instructions. First, 0.5 μg of treated RNA was mixed with 2 μM Oligo (dT) Primer and 1 mM dNTPs; this mix was heated at 65 °C for 10 min and then rapidly placed on ice for 8–10 min.

Table 1
Characteristic of the tomato varieties including registered name, breeding company, collection site, properties, disease severity and resistance reaction levels to Early Blight disease, *Alternaria alternata.*

Genotype No.	Registered name	Origin	Growth Property*	Company	Disease severity/Stages (%)			
					Transplanting		Maturity	
					Means ± SE	Reaction to *A. alternata*	Means ± SE	Reaction to *A. alternata*
1	Ameera RZ	Netherlands	Field	Rex van	18.50^a ± 4.15	Susceptible	80.00^b ± 7.26	Susceptible
2	Caribo	Netherlands	Greenhouse	Golan Seed	16.00^b ± 2.45	Susceptible	47.50^f ± 5.83	Susceptible
3	CH American	US	Field	Us Agri. Seed	11.50^g ± 2.36	Susceptible	87.50^a ± 4.17	Susceptible
4	Chief Falat	Iran	Field	Falat	13.50^e ± 3.05	Susceptible	47.50^f ± 5.83	Susceptible
5	Esfahan local	Iran	Field	Pakan Bazr**	5.50^I ± 0.50	Resistant	18.00^k ± 2.90	Resistant
6	H.a.s 2274	US	Greenhouse	Elit	5.00^I ± 0.00	Resistant	40.00^h ± 5.53	Resistant
7	Hedieh	France	Field	Vilmoren	18.50^a ± 4.15	Susceptible	80.00^b ± 6.24	Susceptible
8	Rio Grande	US	Greenhouse	Gardesco	7.50^j ± 2.00	Resistant	22.50^j ± 6.51	Resistant
9	Shiraz local	Iran	Field	Danab	7.50^j ± 2.00	Resistant	55.00^e ± 6.24	Susceptible
10	Turkish Cherry	France	Field	Vilmoren	6.50^k ± 0.76	Resistant	25.00^j ± 7.07	Resistant
	CV				29.55		52.36	

35 tomato genotypes were treated with fungal pathogen (A. alternata) and their disease severity was determined as presented in methods section (2.2). Statistical analysis of the mean disease severity categorized 35 tested genotypes in three main resistance levels; resistant (Esfahan local, H.a.s 2274, Turkish Cherry, Rio Grande, Shiraz local (only in transplant stage)) , tolerant (111 Falat American, Rayosol RZ, Roma, Italian Round, Valouro RZ) and susceptible (Aryia Netherlands, CH American, Caribo, CH, Hyb. Retinto, Izmir, Ameera RZ, Hedieh, Super Cristal American, H.a.s 2194, Hyb. Sogno, Hyb. Retinto, Chief Falat, 111 Falat, Granisum Romania, Hyb. Belize, Hybrid Cherry, Hyb Prolico, Hyb. Santawest, Hyb. SV 1201, Hyb. Ventero, Long Tomato Y, Sakhia, Super Chief American, Supermobile, Super Chief).

*Growth Property, indicating the ideal growing places of the tomato genotypes that were intended for field and or greenhouse. Nevertheless, the trials were conducted in a greenhouse for all uniformly.

**Pakan Bazr Company, Isfahan, Iran. The values followed by the same letter in the column are not differ significantly (0.01%) in Duncan's multiple range test.

Fig. 1. A and B, The site of experiments showing variations on tomato genotypes in response to early blight, *Alternaria alternate*; C and D, the typical symptoms of the disease on tomatoes leaves.

Then, 4 μl RT buffer (5 ×) and 1 μl (200 unit) of reverse transcriptase were added to each tube and incubated at 50 °C for 50 min followed by a 15 min incubation at 72 °C to stop cDNA synthesis (McNeece et al., 2019; Upadhyay et al., 2014).

2.5. Primer design

In this study, the primer sequences utilized in a previous study by Roylawar et al. (2015) were used (Table 2). Adjustment of optimum annealing temperature for each primer pairs was performed by gradient

PCR. Based on the melting temperature (Tm) of each primer, five different temperatures were predicted for each primer pairs, and the PCR was performed on Bio-Rad system (T100™ Thermal Cycler, USA) and programmed for initial denaturation at 95 °C for 3 min followed by 45 cycles of 10 s at 95 °C, 20 s at five different temperatures for each primer pairs and 35 s at 72 °C. The reaction products were run using a TBE agarose gel and, based on the best amplification product, the most suitable annealing temperature was selected (Shinde et al., 2018).

2.6. Real-time PCR conditions

Real-time PCR was performed using RB S3p SYBR Green Master Mix (2 ×) (RNA Biotech, Co) and Applied Biosystems™ StepOne™ Real-Time PCR System (Thermo Fisher Scientific, ABI, USA), according to the manufacturer's instructions and Quantitation-Comparative Ct (ΔΔCt) method. The reaction mixture was prepared with 12.5 μl RB S3p SYBR Green Master Mix, 0.5 μM of each primer, and 100 ng of cDNA, and the final volume was adjusted to 25 μl with sterile deionized water. QRT-PCR was programmed for initial denaturation at 95 °C for 5 min and denaturation at 95 °C for 15 s, specific annealing temperature for each primer pair (Table 2) for 20 s, and extension at 72 °C for 30 s, all for 45 cycles. All experiments were carried out in triplicate regarding the biological samples, and in duplicate regarding the technical procedures. In order to verify the amplified products, a melting curve analysis was performed (Lawaju et al., 2018). The relative changes in the gene expression level in the cDNA samples examined was calculated using the following equation:

$$ratio = \frac{E_{target}^{\Delta Ct(control-sample)}}{E_{ref}^{\Delta Ct(control-sample)}}$$

In this equation, E_{target} is the efficiency of primers of target genes, the E_{ref} is the efficiency of internal control gene, and Ct is the threshold cycle of each of the cDNA samples. In this study, ef1α gene was used as an internal control for the normalization of gene expression data. The efficiency of each primer pair was obtained by LinRegPCR software version 2012.3 (Ruijter et al., 2009) and the threshold cycle (Ct) values for each cDNA were obtained from StepOne (v. 2.3, Thermo Fisher Scientific, ABI) software.

Table 2
Primer sequences of selected genes and Real-Time PCR Amplification Parameters.

Gene Name		Forward and Reverse primers sequence (5' - 3')	GC%	Annealing temperature. (°C)	Aamplicon size (bp)	[a]Accession No.
SlWRKY1	F	TAGCAGTGAAGTGGATGTAGTC	45.45	55	155	XM_019214874.2
	R	TTGGATTATGGGATGACCTCTC	45.45			
SlWRKY11	F	GTAGTGTTAGAGGCTGTCCG	55	55.2	165	NM_001321616.1
	R	AAGCCATCATTCTCTCGCC	52.63			
SlWRKY39	F	GCAAGTGGTCAGAAGCGTAA	50	55.4	127	XM_026029782.1
	R	AGGCTGCTTGAAAACTTGG	47.37			
SlWRKY53	F	GGCTATTCACCGTCTTTTG	47.37	53.8	156	XM_004244582.4
	R	CGTTGTGGAAGTGTTGGC	55.56			
SlWRKY70	F	GCATCATCATCCTCGTCCA	52.63	56.7	117	XM_019214011.2
	R	CACGACCGCAACAACCAT	55.56			
SlNPR1	F	GGGATAGTGGAAGGAAGTG	52.63	53	176	CP023760.1s
	R	GATTTCGTAAGTGCCTCCT	47.37			
PDF1.2	F	CACTTCACAAATGTCGATCCG	47.62	56.5	149	XR_740886.3
	R	AGCCAAATCCAATGCAGTCTC	47.62			
SlPR1α	F	GGTAACTGGAGAGGACAACG	55	54.5	170	NM_001247429.1
	R	GTCACATAAGCATAGCCTGG	50			
SlPR2	F	GCTACTGAGAAACTTGGAGG	50	53	167	NM_001247869.2
	R	GTCCTTCCTGGTTTCTTTG	47.37			
SlPR3 ACHI	F	CGGTCAAACATCTCACGAA	47.37	53.7	321	NM_001247475.2
	R	GCTGCTGTATCTTTAGGGG	52.63			
SlPR3 BCHI	F	GCCTTCTTGTCACGATGTC	52.63	54	203	NM_001247474.2
	R	TTTCACCTGGGCTAACTC	50			
SlPR7	F	ATGTCTTGCCCTCACCTTA	47.37	55	159	NM_001247053.2
	R	GATGTCAGCAGGAAGGAGC	57.89			
ef1α	F	GGCCATCAGACAAACCACTC	55	55	184	XM_004240531.4
	R	TCCTGGAGAGCTTCGTG	58.82			

[a] The accessions are presented in https://www.ncbi.nlm.nih.gov/nuccore/?term=; An. tem.: Annealing temperature; Am: Amplicon, GC%: Guanine-Cytosine content percentages.

2.7. Estimation of defense-related enzyme activities on infected leaves

To obtain the enzymatic extract of β-1,3-glucanase, the preweighed infected and non-infected leaves were homogenized in 1.5 ml of 0.05 M (pH 5.5) Na-acetate buffer. The homogenate was centrifuged at 14 000 g for 20 min at 4 °C, as described by Abeles and Forrence (1970) with some modifications. To determine β-1,3-glucanase activity, spectrophotometry at 500 nm was used to assess the occurrence of a catalyzed reaction, using laminarin (Sigma L-9634) as the substrate and the di-nitrosalicylic acid (DNS) method (Miller, 1959). The β-1,3-glucanase enzyme activity was expressed as the amount of glucose per minute per milligram of soluble protein.

Chitinase extraction was performed as per the method described by Jin et al. (2005) with some modifications. The preweighed infected and non-infected leaves were homogenized in 3 ml of 0.1 M (pH 5.5) Na-acetate buffer. The homogenate was centrifuged at 13 000 g for 15 min at 4 °C. Chitinase activity was measured spectrophotometrically (582 nm) by incubation with colloidal chitin (Miller, 1959). Chitinase activity was determined by DNS method using N-acetyl glucosamine (GlcNAc) as the standard. The chitinase activity is defined as 1 unit activity according to the liberation of 1 μmol of GlcNAc per hour. The total protein content of each sample was determined in 0.1 g fresh leaf by the Bradford procedure (1976) using bovine serum albumin as the standard.

2.8. Statistical analysis

All experiments in this study were performed in a completely randomized design. In the plant pathogenicity experiments, three biological replications were considered for each test specimen, and in molecular studies for each sample, three biological replications and two technical replications were considered. One-way ANOVA was used to analyze the variance of the data and the mean comparison of the data was evaluated using the Duncan test. Statistical analysis and calculations were performed using SPSS version 16.0 and the statistical significance level of the data was calculated at 5% ($P \leq 0.05$) (Nasr Esfahan, 2019).

3. Results

3.1. Disease severity evaluation

Data analysis of the disease severity in both development stages, transplanting and maturing, categorized 35 genotypes into three levels of resistance (as highlighted in Table 1 footnotes). Disease severity percentage of tomato varieties was between 5 and 20% at transplanting stage, and 18–88% at maturing stage (Table 1 and Fig. 1). At the transplanting stage, the selected resistant varieties were H.a.s 2274, Esfahan Local, and Shiraz Local, and the susceptible varieties were Caribo, Hedieh, and Ameera RZ, whereas at the maturing stage, the selected resistant varieties were Rio Grande, Turkish Cherry, H.a.s 2274, and Esfahan local, and the selected susceptible varieties were Chief Falat, CH American, Hedieh, and Ameera RZ. In both the development stages, H.a.s 2274 and Esfahan local exhibited significant resistance to tomato EB disease. Indeed, Hedieh and Ameera RZ showed susceptibility to EB at both growth stages (Table 1 and Fig. 1A and B).

3.2. Comparative expression pattern analysis of SlWRKYs

The qRT-PCR analysis displayed significant changes in SlWRKYs expression between pathogen-inoculated resistant and susceptible genotypes during the transplanting and maturing stages (Fig. 2C–G). In the transplanting stage, all genotypes showed significant upregulation in gene expression of SlWRKY1 as compared to control plants, but the highest expression was observed in H.a.s 2274 (up to 10.7-fold) and Caribo (up to 2.1-fold), whereas a modest upregulation in Esfahan Local variety (up to 0.74-fold) was found under treatment of *A. alternata*. In the maturing stage, the highest significant upregulation of SlWRKY1 gene was achieved in Esfahan Local variety, H.a.s 2274, and Rio Grande, being up to 21.4-, 12-, and 4.2-fold, respectively (Fig. 2C). In the transplanting stage, the expression level of WRKY11 gene was significantly elevated after inoculation with *A. alternata* in the leaves of resistant genotypes H.a.s 2274 (15.9-fold), Esfahan Local variety (0.87-fold), and Shiraz Local variety (0.91-fold), as well as in the susceptible genotype Hedieh (0.73-fold), as compared to other genotypes (Fig. 2D).
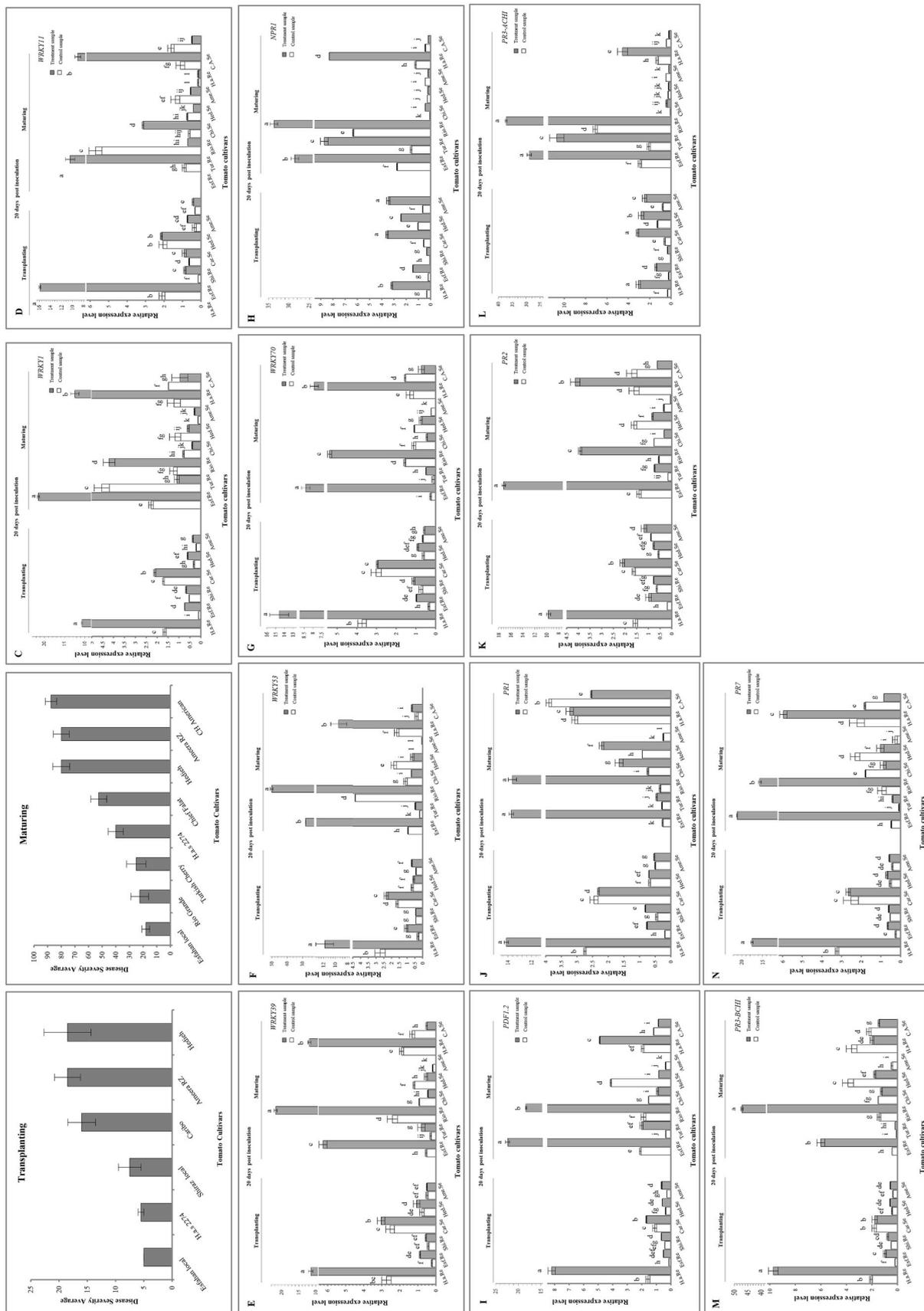
Fig. 2. A and B; Disease severity percentage of six studied tomato varieties at transplanting stage and eight studied tomato varieties at flowering stage after leaves-plant inoculation with A. alternata. The disease severity percentage is determined by the disease severity as described by Nasr Esfahani et al. (2012). C–N; Comparative analysis of genes expression in six genotype in 20 days post inoculation (dpi) and in eight genotype in 20 dpi. Re: Resistant, Se: Sensitive. Genotype names: H.a (H.a.s 2274), Esf (Esfahan local variety), Shi (Shiraz local variety), Car (Caribo), Hed (Hedieh), Ame (Ameera RZ), Tur (Turkish Cherry), Rio (Rio Grande), Chi (Chief Falat) and C.A (CH American). Gene expression is indicated as a relative expression value compared with control in seedlings and maturing. E*f1α* was used as a endogenous control in these experiments. Different letters indicate statistically significant differences (*P* ≤ 0.05).

In the maturing stage, the transcripts of SlWRK11 were significantly higher in resistant plants such as Esfahan Local variety, H.a.s 2274, and Rio Grande (10.4-, 9-, and 3.1-fold, respectively) in response to *A. alternata* (Fig. 2D). In the transplanting stage, the expression of SlWRKY39 gene was strongly upregulated in the inoculated resistant genotypes such as H.a.s 2274 and Esfahan Local variety (11.36- and 0.87-fold, respectively) and the susceptible genotype Caribo (3-fold) (Fig. 2E). In the maturing stage, all inoculated resistant genotypes showed significant upregulation in SlWRKY39 gene expression when compared to control plants, but the strongest was observed in Rio Grande, H.a.s 2274, and Esfahan Local variety (21.6-, 11.7-, and 6.2-fold, respectively; Fig. 2E). In the transplanting stage, the expression level of SlWRKY53 gene was significantly upregulated in H.a.s 2274 (11.83-fold), Esfahan Local variety (1.06-fold), and Caribo (2.3-fold) as compared to control plants (Fig. 2F). In the maturing stage, the highest significant upregulation of SlWRKY53 was observed in varieties carrying resistant genotypes such as Rio Grande, Esfahan Local variety, and H.a.s 2274 (50-, 28.08- and 9.87-fold, respectively) under *A. alternata* stress (Fig. 2F). In the transplanting stage, the expression level of SlWRKY70 gene was significantly upregulated in all inoculated resistant genotypes: H.a.s 2274 (14.57-fold), Esfahan Local variety (0.97-fold), and Shiraz Local variety (1.11-fold), compared with susceptible ones (Fig. 2G). In addition, in the maturing stage, all inoculated resistant genotypes showed significant expression of SlWRKY70 gene but higher expression was observed in Esfahan Local variety, H.a.s 2274, and Rio Grande (8.4-, 7.93-, and 5.38-fold, respectively) than in the susceptible varieties (Fig. 2G).

3.3. Comparative expression analysis pattern of PR genes

The evaluation of expression levels of seven PR genes post inoculation with *A. alternata* revealed that not only these genes were expressed in all genotypes, but also were significantly induced in resistant genotypes at two growth stages. To assess differences in PR expression levels between tomato genotypes, transcriptomes were compared to control plants at both stages (Fig. 2H–N).

In the transplanting stage, upregulation of NPR1, which encodes Non-expressor of Pathogenesis-Related gene1, was observed in most inoculated genotypes but the highest expression was observed in Caribo (3.56-fold), Ameera RZ (3.47-fold), H.a.s 2274 (3.17-fold), Hedieh (2.42-fold), and Esfahan local variety (1.45-fold) as compared to control genotypes (Fig. 2H). In the maturing stage, the expression of NPR1 was strongly elevated in all resistant genotypes such as Rio Grande, Esfahan local variety, Turkish Cherry, and H.a.s 2274 (33-, 27.7-, 8.7-, and 8.3-fold, respectively) in response to *A. alternata* infection (Fig. 2H). In the transplanting stage, the expression of PDF1,2, which encodes a defensin protein, was upregulated in all inoculated genotypes, with the highest expression observed in H.a.s 2274 (8.19-fold) as an inoculated resistant plant and Caribo (1.68-fold) compared to other genotypes (Fig. 2I). In the maturing stage, the expression of PDF1,2 was significantly upregulated in all resistant genotypes, Esfahan local variety (22.78-fold), Rio Grande (18.77-fold), H.a.s 2274 (4.89-fold), and Turkish Cherry (2.02-fold) in response to infection as compared to other plants (Fig. 2I).

In the transplanting stage, the expression level of PR1 gene, encoding pathogenesis-related protein 1, was distinctly altered in all the inoculated resistant genotypes H.a.s 2274, Esfahan local, and Shiraz Local (14.11-, 0.81- and 0.76-fold, respectively) as compared to other plants (Fig. 2J). In the maturing stage, the expression of the PR1 gene was elevated in all inoculated resistant genotypes, but the highest expression was observed in Esfahan local (13.67-fold), Rio Grande (13.58-fold), and H.a.s 2274 (3.23-fold), while the upregulation of PR1 gene was observed in the susceptible varieties, Hedieh (1.65-fold) and Chief Falat (2.21-fold), in response to *A. alternata* infection (Fig. 2J). In the transplanting stage, the transcripts of PR2 gene, encoding β-1,3-glucanase, were significantly elevated in most inoculated genotypes but the

highest expression level was achieved in H.a.s 2274, Caribo, and Esfahan local (9.7-, 2.11-, and 0.99-fold, respectively) as compared to their controls (Fig. 2K). In the maturing stage, the upregulation of PR2 gene expression was observed in all inoculated resistant genotypes, Esfahan local (17.11-fold), H.a.s 2274 (4.13-fold), Rio Grande (3.92-fold), and Turkish Cherry (0.73-fold) as compared to other genotypes (Fig. 2K).

In the transplanting stage, all inoculated genotypes showed significant expression level of PR3-ACHI gene, which encodes acidic chitinase, but the highest was observed in Caribo, H.a.s 2274, Hedieh, Ameera RZ, and Esfahan local (3.13-, 3.06-, 2.8-, 2.49-, and 1.41-fold, respectively) as compared to control plants (Fig. 2L). In the maturing stage, the strongest expression level of PR3-ACHI gene was observed in all inoculated resistant genotypes, Rio Grande (37.19-fold), Esfahan local (28.98-fold), Turkish Cherry (10.69-fold), and H.a.s 2274 (4.54-fold) compared to control plants (Fig. 1L). In the transplanting stage, a significant accumulation of transcripts of PR3-BCHI gene, which encodes basic chitinase, was recorded in H.a.s 2274 (9.92-fold) and Esfahan local (0.99-fold) upon *A. alternata* infection (Fig. 2M). In the maturing stage, significant upregulation of PR3-BCHI gene was observed only in Rio Grande and Esfahan local (47.04- and 6.12-fold, respectively) in response to *A. alternata* infection as compared to control plants. However, PR3-BCHI gene expression was downregulated or remained unchanged in other genotypes (Fig. 2M).

At the transplanting stage, the expression of PR7 was significantly upregulated in inoculated resistant genotypes, H.a.s 2274 and Esfahan local variety (17.51- and 0.65-fold, respectively), while that of PR7 gene remained unchanged in other inoculated genotypes (Fig. 2N). In the maturing stage, the transcription level of PR7 gene was significantly increased in all resistant genotypes by up to 21.11-, 15.63-, 5.95-, and 0.4-fold in Esfahan local, Rio Grande, H.a.s 2274, and Turkish Cherry, respectively, as compared to the control plants. However, PR7 gene expression was downregulated in all inoculated susceptible genotypes as compared to their controls in response to *A. alternata* (Fig. 2N).

3.4. Comparative changes in β-1,3-glucanase and chitinase activities

The results of total protein quantification at both growth stages showed a significant increase in most inoculated tomato leaves as compared with controls (Table 3 and Table 4). The results on the effect of *A. alternata* in the activity of β-1,3 glucanase and chitinase enzymes showed that the activity of these enzymes was considerably increased in all inoculated resistant genotypes compared to their controls at the transplanting and maturing stages (Tables 3 and 4). The specific activity analysis of β-1,3-glucanase and chitinase at the transplanting stage indicated the highest changes in Shiraz local genotype (0.4 U/mg and 0.12 U/mg, respectively) among all inoculated genotypes (Table 3). Maximum specific activity of β-1,3 glucanase and chitinase at the maturing stage was found in Turkish Cherry (0.51 U/mg and 0.24 U/mg, respectively) after *A. alternata* inoculation (Table 4). Our results indicate that there was a significant correlation between the expression levels of PR2 and PR3 genes along with a high accumulation of the gene transcription with the specific enzymatic activity of chitinase and glucanase in tomato EB resistance in most selected resistant varieties (Table 5).

4. Discussion

This is the first report on the resistance of tomato genotypes against *A. alternata* and the molecular basis for the defense mechanisms, which involve the activity of 12 genes in the resistant varieties. Our investigations on the resistance of 35 screened tomato genotypes showed remarkable and different molecular reactions in response to *A. alternata* at two different growth stages. The results showed that the differences among genotypes regarding the severity of disease was mainly due to the genetic variation within the studied population. Unfortunately,

Table 3
Determination of β-1,3 Glucanase and Chitinase activities in inoculated tomato leaves as compared to the controls at transplanting stage to Early Blight disease, *Alternaria alternata*.

Genotype No.	Selected Genotypes	Total Protein mg/m		β-1,3-glucanase Activity U/ml.min		Specific β-1,3-glucanase Activity U/mg		Chitinase Activity U/ml.h		Chitinase Specific Activity U/mg		Reaction to A. alternata R/S
		Control	Treat	Control	Treat	Control	Treat	Control	Treat	Control	Treat	
1	Ameera RZ	$1.45^d \pm 0.08$	$1.44^d \pm 0.06$	$0.16^e \pm 0.02$	$0.18^g \pm 0.07$	$0.11^{fg} \pm 0.016$	$0.07^h \pm 0.00$	$0.04^d \pm 0.00$	$0.05^d \pm 0.01$	$0.02^f \pm 0.00$	$0.03^{ef} \pm 0.00$	S
2	Caribo	$0.94^f \pm 0.06$	$1.23^e \pm 0.06$	$0.12^{fg} \pm 0.00$	$0.26^c \pm 0.01$	$0.12^f \pm 0.00$	$0.21^d \pm 0.00$	$0.05^d \pm 0.00$	$0.06^{cd} \pm 0.01$	$0.05^d \pm 0.01$	$0.05^d \pm 0.00$	S
3	Esfahan local	$1.64^c \pm 0.10$	$2.09^b \pm 0.03$	$0.15^{ef} \pm 0.01$	$0.65^b \pm 0.01$	$0.09^g \pm 0.01$	$0.31^b \pm 0.00$	$0.09^c \pm 0.06$	$0.18^b \pm 0.00$	$0.04^{de} \pm 0.01$	$0.09^b \pm 0.00$	R
4	H.a.s 2274	$1.43^d \pm 0.13$	$3.08^a \pm 0.04$	$0.06^h \pm 0.01$	$0.85^a \pm 0.06$	$0.04^i \pm 0.01$	$0.28^c \pm 0.01$	$0.07^{cd} \pm 0.00$	$0.31^a \pm 0.02$	$0.05^d \pm 0.01$	$0.01^b \pm 0.01$	R
5	Hedieh	$1.69^c \pm 0.12$	$2.03^b \pm 0.08$	$0.21^d \pm 0.02$	$0.09^{gh} \pm 0.01$	$0.12^f \pm 0.00$	$0.04^k \pm 0.00$	$0.07^{cd} \pm 0.01$	$0.05^{cd} \pm 0.01$	$0.04^{de} \pm 0.01$	$0.03^f \pm 0.01$	S
6	Shiraz local	$0.62^g \pm 0.03$	$1.62^d \pm 0.088$	$0.12^{fg} \pm 0.01$	$0.64^b \pm 0.01$	$0.12^e \pm 0.00$	$0.4^a \pm 0.02$	$0.04^d \pm 0.01$	$0.2^b \pm 0.02$	$0.07^c \pm 0.01$	$0.12^a \pm 0.01$	R

Comparative analysis of total protein, β-1,3-Glucanase activity, Chitinase Activity and Chitinase specific activity in six genotypes in 20 days post inoculation (dpi) at transplanting stage. U:1 mmol glucose or 1 µmol of GlcNAc released by β-1,3-Glucanase or Chitinase activities, respectively, R: Resistant, S: Sensitive. Each value represents the mean of three replicates.

Table 4
Quantification of β-1,3 Glucanase and Chitinase activities in inoculated tomato leaves as compared to the controls at maturing stage to Early Blight disease, *Alternaria alternata*.

Genotype No.	Registered name	Total Protein mg/ml <		β-1,3-Glucanase Activity U/ml.min		β-1,3-Glucanase Specific Activity U/mg		Chitinase Activity U/ml.h		Chitinase Specific Activity U/mg		Reaction to A. alternata R/S
		Control	Treat	Control	Treat	Control	Treat	Control	Treat	Control	Treat	
1	Ameera RZ	$1.24^{fg} \pm 0.06$	$1.35^{ef} \pm 0.07$	$0.3^e \pm 0.01$	$0.23^f \pm 0.01$	$0.23^e \pm 0.00$	$0.17^{fg} \pm 0.00$	$0.09^{gh} \pm 0.01$	$0.07^{hij} \pm 0.01$	$0.07^f \pm 0.01$	$0.05^g \pm 0.01$	S
2	CH American	$0.54^k \pm 0.08$	$1.28^g \pm 0.05$	$0.1^i \pm 0.02$	$0.28^g \pm 0.02$	$0.19^f \pm 0.01$	$0.16^g \pm 0.02$	$0.08^{ghi} \pm 0.01$	$0.08^{fgh} \pm 0.01$	$0.15^d \pm 0.00$	$0.07^f \pm 0.01$	S
3	Chief Falat	$1.02^i \pm 0.12$	$1.7^{cd} \pm 0.06$	$0.15^h \pm 0.01$	$0.13^{hi} \pm 0.01$	$0.15^{gh} \pm 0.02$	$0.07^j \pm 0.01$	$0.11^{ef} \pm 0.01$	$0.05^j \pm 0.01$	$0.11^e \pm 0.02$	$0.03^j \pm 0.01$	S
4	Esfahan Local Variety	$1.75^c \pm 0.04$	$2.05^b \pm 0.06$	$0.2^g \pm 0.02$	$0.6^c \pm 0.01$	$0.11^l \pm 0.01$	$0.29^d \pm 0.00$	$0.25^d \pm 0.01$	$0.44^b \pm 0.03$	$0.14^c \pm 0.01$	$0.21^b \pm 0.01$	R
5	H.a.s 2274	$0.85^j \pm 0.06$	$1.45^e \pm 0.06$	$0.11^i \pm 0.00$	$0.65^b \pm 0.02$	$0.13^{ij} \pm 0.01$	$0.45^b \pm 0.01$	$0.1^{efg} \pm 0.02$	$0.23^d \pm 0.01$	$0.12^e \pm 0.02$	$0.16^d \pm 0.00$	R
6	Hedieh	$1.16^{gh} \pm 0.06$	$1.1^b \pm 0.1$	$0.28^e \pm 0.001$	$0.18^e \pm 0.02$	$0.25^e \pm 0.02$	$0.09^e \pm 0.01$	$0.06^{ij} \pm 0.01$	$0.05^j \pm 0.01$	$0.05^g \pm 0.00$	$0.02^b \pm 0.00$	S
7	Rio Grande	$1.08^{hi} \pm 0.05$	$2.6^a \pm 0.03$	$0.12^{hi} \pm 0.01$	$0.36^d \pm 0.01$	$0.12^j \pm 0.01$	$0.14^{hi} \pm 0.01$	$0.12^e \pm 0.01$	$0.48^a \pm 0.01$	$0.11^e \pm 0.01$	$0.19^c \pm 0.01$	R
8	Turkish Cherry	$0.36^l \pm 0.04$	$1.62^d \pm 0.06$	$0.11^l \pm 0.01$	$0.82^a \pm 0.03$	$0.32^c \pm 0.02$	$0.51^a \pm 0.01$	$0.07^{hij} \pm 0.01$	$0.4^c \pm 0.01$	$0.18^c \pm 0.01$	$0.24^a \pm 0.01$	R

Activity of Total Protein, β-1,3-Glucanase activity, β-1,3-Glucanase specific activity, Chitinase Activity and Chitinase specific activity in eight genotypes in 20 days post inoculation (dpi). U:1 mmol glucose or 1 µmol of GlcNAc liberated by Chitinase activities, R: Resistant genotype, S: Sensitive genotype. Each value represents the mean of three replicates.

Table 5

The correlation analysis between specific enzyme activity of β-1, 3-Glucanase enzyme and Chitinase with the expression of PR2 and PR3-(ACHI/BCHI) genes in transplanting and maturing stages.

Enzyme Activity	Correlation	Transplanting Stage			Maturing Stage		
		PR2	PR3.ACHI	PR3.BCHI	PR2	PR3.ACHI	PR3.BCHI
β-1,3 Glucanase.	Pearson Correlation	.337[b]	.000	.298	.285[b]	.135	-.154
	Sig.	.045	1.000	.077	.050	.461	.400
Chitinase	Pearson Correlation	.397[b]	-.113	.429[a]	.452[a]	.598[a]	.276
	Sig.	.016	.512	.009	.009	.000	.126

[a] Correlation is significant at the 0.01 level (2-tailed).
[b] Correlation is significant at the 0.05 level (2-tailed).

there are only a few reports describing extensive searches for possible sources of resistance to *Alternaria* species in the tomato germplasm (Adhikari et al., 2017; Nasr Esfahani et al., 2017a; b, 2018).

The disease severity percentage of EB disease among tomato genotypes divided the genotypes into three groups as resistant, tolerant, and susceptible. The screening assay at the transplanting stage resulted in the identification three genotypes as resistant (H.a.s 2274, Shiraz Local, and Esfahan local) and three genotypes as susceptible (Hedieh, Ameera RZ, and Caribo) to *A. alternata* infection, whereas in the one performed at the maturing stage identified four genotypes as resistant (Esfahan local, Rio Grande, H.a.s 2274, and Turkish Cherry) and four genotypes as susceptible (Hedieh, Ameera RZ, CH American, and Chief Falat).

Our results clearly showed that two genotypes, H.a.s 2274 and Esfahan local, were resistant to EB disease at both stages, while the majority of tomato genotypes were susceptible at least at one of the stages. Our screening findings confirmed previous results obtained in screening germplasm collections of tomato, where the majority of genotypes belonged to varieties susceptible to the disease caused by *Alternaria* (Adhikari et al., 2017; Foolad et al., 2000; Singh et al., 2011; Soleimani and Kirk, 2012). In our study, H.a.s 2274 and Esfahan local were the most resistant varieties to the pathogen, with almost the same disease severity rates at the transplanting and maturing stages (40% and 18%, respectively). The results showed that EB disease severity percentage is more pronounced at the maturing stage rather than at the transplanting stage, in agreement with previous reports by Adhikari et al. (2017), Nasr Esfahani et al. (2017a, b), Singh et al. (2011), and Vloutoglou and Kalogerakis (2000).

Based on our results, the expression levels of SlWRKY1 and SlWRKY11 were assessed among all examined genotypes. High accumulation of the gene transcription was observed in most inoculated resistant varieties (H.a.s 2274 and Esfahan local at the transplanting stage, and Esfahan local, Rio Grande, and H.a.s 2274 at the maturing stage). Additionally, a similar gene expression pattern of SlWRKY1 and SlWRKY11 was observed among tomato varieties throughout the experiment. The similarity in expression pattern of these genes can highlight combined potential synergistic effect in the defense mechanism of tomato. A higher expression of WRKY1 in some genotypes, such as H.a.s 2274 and Caribo, than in the other varieties even before inoculation was observed; numerous reports have already established the important roles of WRKY proteins in various physiological processes including seed germination, lateral root formation, flowering, fruit ripening, leaf senescence, and metabolic processes. WRKY proteins also play important regulatory roles in plant defense against various biotic stresses (Yang et al., 2018). In addition, certain plants are resistant at seedling stages, but may not be resistant at maturing stages, as in the case of EB disease caused by *A. alternata* in potatoes (Nasr Esfahani et al., 2017a,b). The results may suggest the antifungal role of these TFs in response to EB, which is in line with a recent study confirming the potential involvement of the overexpression of the WRKY1 gene in the resistance of tomato plants to *Phytophthora infestans* (Cui et al., 2019). In *Solanum arcanum*, Shinde et al. (2018) identified WRKY1 as a positive regulator involved in the expression of defense-related genes,

resulting in enhanced disease resistance against *A. solani*. The upregulation of WRKY11 was also observed in resistant accessions of *S. Arcanum* to *A. solani* Shinde et al. (2018)(. Data comparison between SlWRKY39, SlWRKY53, and SlWRKY70 also showed similar significant expression patterns in all inoculated resistant varieties at the maturing stage and in the majority of inoculated resistant varieties at the transplanting stage. Our results, along with those reported by Bai et al. (2018), confirmed that SlWRKY39 was significantly upregulated in tomato infected by *Pseudomonas syringae*. Furthermore, other studies reported that AtWRKY70 gene expression was altered in response to *Alternaria brassicicola* and *Botrytis cinerea,* and changes in activity of AtWRKY70 might enhance susceptibility to *Erysiphe cichoracearum*, *B. cinerea*, *Macrophomina phaseolina,* and gall formation by *Linaria vulgaris* (Lawaju et al., 2018; Pandey et al., 2016; Ulker et al., 2007; Zorica et al., 2019).

The gene expression data revealed that during plant interaction with fungal pathogens, several WRKY genes were involved in tomato defense response to prevent or minimize the damages caused by pathogen to plant system (Bai et al., 2018). Indeed, some tomato WRKY genes have been characterized as genes with the ability of inducing transcription, and identified to be positive regulators of PRs during fungal pathogen invasion (Bai et al., 2018; Chaudhary and Atamian, 2017). Many studies defined PR genes as host plant genes induced under pathological or related conditions, while PR activity reduces the symptom severity upon pathogen infection (Sudisha et al., 2012). In addition, differential expression of these defense-related genes is regulated not only by SA- and JA-mediated signal pathways, but the gene expression level also varies between resistant and susceptible genotypes to *Alternaria* species (Pathak et al., 2017; Yang et al., 2015).

Our findings demonstrated that similar expression patterns for SlWRKY1 gene was also observed in PR1 and PR2 genes in most selected genotypes, especially in H.a.s 2274, Shiraz Local, Esfahan local, and Rio Grande as resistant plants in response to *A. alternata* at both growth stages. Moreover, the expression patterns of SlWRKY1 and SlWRKY11 were similar to that of PR7 and PDF1.2 genes. Current findings support the possible involvement of these TFs in the regulation and PR gene expression in response to EB. This was also reported previously in other studies, where tomato SlWRKY1 acts as a key component for expression regulator of defense-related genes, including PR1 and PR2 (Bai et al., 2018; Shinde et al.,2018), and the upregulation of SlWRKY11 being positively correlated with the high expression of the PR7 gene (Roylawar et al., 2015; Shinde et al., 2018).

The experiments showed that the expression pattern of SlWRKY genes 39, 53, and 70, was comparable to PR2 and PR7 gene expression in selected genotypes at both stages, indicating the possible regulatory role of these three TFs together with SlWRKY genes 1 and 11 on the expression of the PR genes; this is in line with another study suggesting the positive role of SlWRKY70 in regulating the expression of PR1 (Roylawar et al., 2015; Pandey et al., 2016). In addition to the above findings, in most selected genotypes, the expression pattern of SlWRKY53 was comparable to that of the PR3-BCH gene. However, all the SlWRKY genes exhibited different expression patterns relative to

PR3-BCH expression, suggesting ideas for future investigation toward the identification of specific TF gene(s) involved in the expression of this particular PR. The evaluation of NPR1 and PR1 gene expression in this study also showed their significant upregulation in all the inoculated resistant genotypes at both growth stages. However, enhanced NPR1 expression was observed in inoculated resistant genotypes, Esfahan local, Rio Grande, H.a.s 2274, and Turkish Cherry to *A. alternata.* Several recent studies have shown the NPR1 gene to be a co-TF promoting the expression of PR genes in the systemic signaling of plant defense, particularly the systemic acquired resistance (SAR) (Silva et al., 2018; Wang et al., 2018). Furthermore, our results suggested the potential role of NPR1 gene in the regulation of transcription in response to EB, and its upregulation within all of the inoculated genotypes at the transplanting stage highlights its potential basal defense role in the early stages of infection (Hussain et al., 2018). The results of the present study showed a positive relationship between WRKY1 induction and increased expression of NPR1, and also WRKY1-related PR1 upregulation in all of the plants resistant against *A. alternata.* The expression information of these genes will be useful in further investigating their function under various biotic stress conditions. In some exceptions, the elevated expression levels of PR1 were observed in the inoculated susceptible plants Chief Falat and Hedieh at the maturing stage, suggesting the possible function of PR1 in floral development, which is consistent with findings of Bonasera et al. (2006) on PR gene expression in response to elicitors inoculated by *Erwinia amylovora* on apple.

Our study also revealed the significant upregulation of PDF 1.2 gene expression in all the inoculated resistant tomato plants at the transplanting stage. However, the increased expression level of PDF 1.2 gene was obtained only in the inoculated resistant genotypes H.a.s 2274, Esfahan local, Turkish Cherry, and Rio Grande at maturing stage, suggesting its main involvement in EB disease resistance, occurring in the mature growth stage. This finding is in agreement with those of several studies on antifungal activity of leaf-specific defense in PDF1.2, which plays a significant role in the innate immune response and enhanced plant resistance (De Coninck et al., 2013; Manners et al., 1998; Thomma et al., 2002; Zahirnejad et al., 2018).

Our results showed an enhanced expression of PR2 and acidic PR3 genes in all the inoculated plants at both growth stages, suggesting their key basal role in innate immune response and induced resistance to pathogen infection. The high amount of PR2 and acidic PR3 expression within the inoculated resistant genotypes also highlights its protective role against EB disease, and the increased induction of basic PR3 observed in some inoculated resistant genotypes in both growth stages, may suggest its complementary function with other defense-related genes. Many studies have found PR2 and PR3 induction to be contributors in defense response to fungal invasion (Amaral et al., 2012; Sels et al., 2008; Singh et al., 2014; Van Loon et al., 1994). In particular, within supper Maranda tomato cultivars in comparison to control samples, expression of chitinases was shown to be highly induced upon *Alternaria solani* inoculation (El-Hadary and Tayel, 2013), and synergistic upregulation of PR2 and PR3 in response to *Fusarium oxysporum* in transgenic tomato (Jongedijk et al., 1995) and *Cladosporium fulvum* in resistant tomato plants (Wubben et al., 1996) has also been reported in consistency with these findings.

The findings of the present study showed significant PR7 upregulation within the inoculated resistant variety, H.a.s 2274, at the transplanting stage, and strong expression in the inoculated resistant genotypes, H.a.s 2274, Esfahan local, and Rio Grande, at the maturing stage. In addition, the similarity in its expression pattern to PDF1.2 suggests their combined effect in perception or signaling pathways in EB disease resistance. Therefore, they are considered as key genes involved in the activation of the defense response. PR7, encoding 69endopeptidase, has been identified in tomato plants as a pathogen-induced proteases (Jorda and Vera, 2000; Tornero et al., 1996), its fungal activities determined in a recent study (Golshani et al., 2015), and its expression found in

several pathological situations, including *P. syringae* (Jorda et al., 2000) and *Phytophthora infestans* (Tian et al., 2007) infections.

The analysis of enzyme activity of PR2 and PR3 proteins (β-1,3-glucanase and chitinase) in the tested genotypes suggested possible contribution of plant protection and defense response of both enzymes in tomato EB resistance. The highest enzyme activity of PR2 and PR3 proteins observed in inoculated resistant genotypes may suggest their key role upon EB infection. Many studies showed that the accumulation of chitinase and glucanase as hydrolytic products of constitutive and induced genes affects fungal viability and promotes plant immunity responses (El-Hadary and Tayel, 2013; Kumar et al., 2018; Pusztahelyi, 2018). Zur et. al. (2013) reported that on infection with *Microdochium nivale,* the activity of chitinase was markedly induced in the resistant Polish cultivars of winter triticale, while that of β-1,3 glucanase was not affected by fungus in any of the tested winter triticale cultivars. Our results indicated that there was a significant correlation between the expression levels of PR2 and PR3 genes as a high accumulation of the gene transcription with the specific enzymatic activity of chitinase and glucanase in tomato EB resistance in most selected resistant varieties.

These results sought to shed light on the functional expression of these defense-related genes in EB disease resistance. Among all the inoculated varieties, Esfahan local showed significant resistance and an increased expression of all the defense-related genes at both stages, whereas the Rio Grande variety showed a similar resistance only at the maturing stage. There was also a direct relationship of expression levels of SlWRKY1 and enzymatic activity of chitinase and glucanase in terms of accumulation of the gene transcription in EB-resistant tomato genotypes.

Contributions

Giti Alizadeh Moghaddam: Conducting the project, Obtaining resources, Writing the original draft of the manuscript. Zahra Rezayatmand: Supervision, Review, and Data curation. Mehdi Nasr Esfahani: Supervision, Conceptualization, Methodology, Data curation, Review, and Editing. Mehdi Khozaei: Methodology and Review.

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Conflicts of interest

There is no conflict of interest relating to this article.

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